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[REPLACEMENT SHEET]

Intravascular Delivery of Non-Viral Nucleic Acid

This application is a Continuation-In-Part of the following: U.S. Serial No. 09/450,315 filed on November 29, 1999, U.S. Serial No. 09/000,533 filed on December 30, 1997, U.S. Serial No. 09/070,303 filed on April 30, 1998, U.S. Serial No. 08/571,536 filed on December 13, 1995, and U.S. Serial No. 09/391,260 filed on September 7, 1999, which is a Continuation of Issued Patent No. 6,265,387 filed on December 13, 1995.

Field of the Invention

The invention relates to compounds and methods for use in biologic systems. More particularly, processes that transfer nucleic acids into cells are provided. Nucleic acids in the form of naked DNA or a nucleic acid combined with another compound are delivered to cells.

Background

Biotechnology includes the delivery of a genetic information to a cell to express an exogenous nucleotide sequence, to inhibit, eliminate, augment, or alter expression of an endogenous nucleotide sequence, or to express a specific physiological characteristic not naturally associated with the cell. Polynucleotides may be coded to express a whole or partial protein, or may be anti-sense.

A basic challenge for biotechnology and thus its subpart, gene therapy, is to develop approaches for delivering genetic information to cells of a patient in a way that is efficient and safe. This problem of "drug delivery," where the genetic material is a drug, is particularly challenging. If genetic material are appropriately delivered they can potentially enhance a patient's health and, in some instances, lead to a cure. Therefore, a primary focus of gene therapy is based on strategies for delivering genetic material in the form of nucleic acids. After delivery strategies are developed they may be sold commercially since they are then useful for developing drugs.

Delivery of a nucleic acid means to transfer a nucleic acid from a container outside a mammal to near or within the outer cell membrane of a cell in the mammal. The term transfection is used herein, in general, as a substitute for the term delivery, or, more specifically, the transfer of a nucleic acid from directly outside a cell membrane to within the cell membrane. The transferred (or transfected) nucleic acid may contain an expression cassette. If the nucleic acid is a primary RNA transcript that is processed into messenger RNA, a ribosome translates the messenger RNA to produce a protein within the cytoplasm. If the nucleic acid is a DNA, it enters the nucleus where it is transcribed into a messenger RNA that is transported into the cytoplasm where it is translated into a protein. Therefore if a nucleic acid expresses its cognate protein, then it must have entered a cell. A protein may subsequently be degraded into peptides, which may be presented to the immune system.

It was first observed that the in vivo injection of plasmid DNA into muscle enabled the expression of foreign genes in the muscle (Wolff, J A, Malone, R W, Williams, P, et al. Direct gene transfer into mouse muscle *in vivo*. *Science* 1990;247:1465-1468.). Since that report, several other studies have reported the ability for foreign gene expression following the direct injection of DNA into the parenchyma of other tissues. Naked DNA was expressed following its injection into cardiac muscle (Acsadi, G., Jiao, S., Jani, A., Duke, D., Williams, P., Chong, W., Wolff, J.A. Direct

late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle such as the nucleus. Releasing signals include chemicals such as chloroquine, bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence, SEQ ID 1), viral components such as influenza virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

Cellular receptor signals are any signal that enhances the association of the gene with a cell. This can be accomplished by either increasing the binding of the gene to the cell surface and/or its association with an intracellular compartment, for example: ligands that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with sulfhydryl or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids fatty acids, cholesterol, dansyl compounds, and amphotericin derivatives. In addition viral proteins could be used to bind cells.

Polynucleotides

The term nucleic acid is a term of art that refers to a string of at least two base-sugar-phosphate combinations. (A polynucleotide is distinguished from an oligonucleotide by containing more than 120 monomeric units.) Nucleotides are the monomeric units of nucleic acid polymers. The term includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the form of an oligonucleotide messenger RNA, anti-sense, plasmid DNA, parts of a plasmid DNA or genetic material derived from a virus. Anti-sense is a polynucleotide that interferes with the function of DNA and/or RNA. The term nucleic acids- refers to a string of at least two base-sugar-phosphate combinations. Natural nucleic acids have a phosphate backbone, artificial nucleic acids may contain other types of backbones, but contain the same bases. Nucleotides are the monomeric units of nucleic acid polymers. The term includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). RNA may be in the form of an tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, and ribozymes. DNA may be in form plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition these forms of DNA and RNA may be single, double, triple, or quadruple stranded. The term also includes PNAs (peptide nucleic acids), phosphorothioates, and other variants of the phosphate backbone of native nucleic acids.

A polynucleotide can be delivered to a cell to express an exogenous nucleotide sequence, to inhibit, eliminate, augment, or alter expression of an endogenous nucleotide sequence, or to express a specific physiological characteristic not naturally associated with the cell. Polynucleotides may be coded to express a whole or partial protein, or may be anti-sense.

A delivered polynucleotide can stay within the cytoplasm or nucleus apart from the endogenous genetic material. Alternatively, the polymer could recombine (become a

To a complex of DNA (20 μg/ml) and poly-L-lysine (40 μg/ml) in 1.5 ml was added the various citraconylpolyvinylphenol and citraconylpoly-L-lysine (150 μg/ml). The sizes of the particles formed were measured to be 90-120 nm and the zeta potentials of the particles were measured to be –10 to –30 mV (Brookhaven ZETA PLUS$_{TM}$ Particle Sizer).

To each sample was added acetic acid to make the pH 5. The size of the particles was measured as a function of time. Both citraconylpolyvinylphenol and citraconylpoly-L-lysine DNA complexes were unstable under acid pH. The citraconylpolyvinylphenol sample had particles > 1 μm in 5 minutes and citraconylpoly-L-lysine sample had particles > 1 μm in 30 minutes.

Synthesis of Glutaric Dialdehyde – Poly-Glutamic acid (8mer) Copolymer

H_2N-EEEEEEEE-$NHCH_2CH_2NH_2$ (SEQ ID 2; 5.5 mg, 0.0057 mmol, Genosys) was taken up in 0.4 ml H_2O. Glutaric dialdehyde (0.52 μL, 0.0057 mmol, Aldrich Chemical Company) was added and the mixture was stirred at room temperature. After 10 min the solution was heated to 70 °C. After 15 hrs, the solution was cooled to room temperature and dialyzed against H_2O (2x2L, 3500 MWCO). Lyophilization afforded 4.3 mg (73%) glutaric dialdehyde-poly-glutamic acid (8mer) copolymer.

Synthesis of Ketal from Polyvinylphenyl Ketone and Glycerol

Polyvinyl phenyl ketone (500 mg, 3.78 mmol, Aldrich Chemical Company) was taken up in 20 ml dichloromethane. Glycerol (304 μL, 4.16 mmol, Acros Chemical Company) was added followed by p-toluenesulfonic acid monohydrate (108 mg, 0.57 mmol, Aldrich Chemical Company). Dioxane (10 ml) was added and the solution was stirred at room temperature overnight. After 16 hrs, TLC indicated the presence of ketone. The solution was concentrated under reduced pressure, and the residue redissolved in DMF (7 ml). The solution was heated to 60° C for 16 hrs. Dialysis against H_2O (1x3L, 3500 MWCO), followed by Lyophilization resulted in 606 mg (78%) of the ketal.

Synthesis of Ketal Acid of Polyvinylphenyl Ketone and Glycerol Ketal

The ketal from polyvinylphenyl ketone and glycerol (220 mg, 1.07 mmol) was taken up in dichloromethane (5 ml). Succinic anhydride (161 mg, 1.6 mmol, Sigma Chemical Company) was added followed by diisopropylethyl amine (0.37 ml, 2.1 mmol, Aldrich Chemical Company) and the solution was heated at reflux. After 16 hrs, the solution was concentrated, dialyzed against H_2O (1x3L, 3500 MWCO), and lyophilized to afford 250 mg (75%) of the ketal acid.

Particle Sizing and Acid Lability of Poly-L-Lysine/ Ketal Acid of Polyvinylphenyl Ketone and Glycerol Ketal Complexes

Particle sizing (Brookhaven Instruments Corporation, ZETA PLUS$_{TM}$ Particle Sizer, I90, 532 nm) indicated an effective diameter of 172 nm (40 μg) for the ketal acid Addition of acetic acid to a pH of 5 followed by particle sizing indicated a increase in particle size to 84000.

A poly-L-lysine/ ketal acid (40 μg, 1:3 charge ratio) sample indicated a particle size of 142 nm. Addition of acetic acid (5 μL, 6 N) followed by mixing and particle sizing indicated an effective diameter of 1970 nm. This solution was heated at 40° C. particle sizing indicated a effective diameter of 74000 and a decrease in particle counts.

were injected into the tail vein of a 25 g ICR mouse (Harlan Sprague Dawley, Indianapolis, IN) in 7 seconds. Animals were sacrificed after 24 hours and livers were removed and assayed for luciferase expression.

Complex Preparation (per mouse)

Complex I: pDNA (pCI Luc, 10 μg) in 2.5 ml Ringers.
Complex II: pDNA (pCI Luc, 10 μg) was mixed with cationic peptide (18 mer KLLKKLLKLWKKLLKKLK; SEQ ID 3) at a 1:2 ratio. Complexes were diluted to 2.5 ml with Ringers solution.

Tail vein injections of 2.5 ml of the complex were preformed as previously described. Luciferase expression was determined as previously shown.

Results: 2.5 ml injections
Complex I: 1.63×10^{10} Relative Light Units per liver
Complex II: 2.05×10^{10} Relative Light Units per liver

Negatively Charged Complexes Using Labile polymers

Delivery of PEI/DNA and histone H1/DNA particles to rat skeletal muscle via intravascular injection into an artery.

Experimental Protocol and Methods:

PEI/DNA and histone H1/DNA particles were injected into rat leg muscle by either a single intra-arterial injection into the external iliac [see Budker et al. Gene Therapy, 5:272, (1998)]. Harlan Sprague Dawley (HSD SD) rats were used for the muscle injections. All rats used were female and approximately 150 grams and each received complexes containing 100 micrograms of plasmid DNA encoding the luciferase gene under control of the CMV enhancer/promoter (pCILuc) [see Zhang et al. Human Gene Therapy, 8:1763, (1997)].

Luciferase Assays:

Results of the rat injections are provided in relative light units (RLUs) and micrograms (μg) of luciferase produced. To determine RLUs, 10 μl of cell lysate were assayed using a EG&G Berthold LB9507 luminometer and total muscle RLUs were determined by multiplying by the appropriate dilution factor. To determine the total amount of luciferase expressed per muscle we used a conversion equation that was determined in an earlier study [see Zhang et al. Human Gene Therapy, 8:1763, (1997)] [pg luciferase = RLUs x 5.1×10^{-5}]

Intravascular Delivery (IV Muscle)

DNA/PEI particles (1 : 0.5 charge ratio)

Muscle Group	*Total RLUs*	*Total Luciferase*
muscle group 1 (upper leg anterior)	3.50×10^9	0.180 μg
muscle group 2 (upper leg posterior)	3.96×10^9	0.202 μg
muscle group 3 (upper leg medial)	7.20×10^9	0.368 μg